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13. ABSTRACT (Maximum 200) Combinatorial phage technology is used to identify and characterize newly expressed proteins which elicit plasma cell reactions in certain breast carcinomas. It is our principal hypothesis that this reaction reflects a local immune response against tumor that is etiologic in the historical impression of a more favourable natural course of these tumors after surgery-only therapy. DNA sequencing of IgG H and L chains from random unselected clones indicates a dramatic focus in libraries derived from two patients with medullary ductal carcinoma, supporting our hypothesis of a local immune response in these tumors. Reactive phage clones are selected from phage display libraries using optimal cell-based panning conditions with medullary ductal carcinoma cells. It was demonstrated that Her2/neu and p53 are not the eliciting antigens. We will be employing immunoprecipitation and immunoblotting in order to isolate the antigen of interest.				
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FOREWORD

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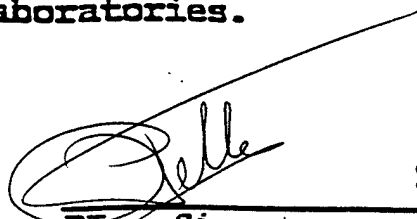
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INTRODUCTION

Among non-hematologic malignancies, immunotherapy has had its most promising application in melanoma, neuroblastoma and renal cell carcinoma [1,2,3]. Other solid tumors, including breast cancer, have been less responsive. The responsiveness of these three cancers (melanoma, etc.) during immunotherapies correlates with anecdotes of long term stabilization or rare spontaneous regressions in which an apparent triggering of the host immune system results in suppression of the tumor. Accordingly, there is a powerful rationale to support such therapies where the natural history of the disease suggests an immunoresponsive component. By this criterion, one stands out as remarkable among all breast cancers: medullary ductal carcinoma (MC).

Medullary carcinomas are diagnosed in up to 5-7% of breast cancers. Grossly, they are circumscribed without encapsulation and are rarely bilateral. MC is also circumscribed microscopically, but its appearance is otherwise highly ominous, with large cells, abundant cytoplasm, large bizarre nuclei and frequent mitoses. Virtually all are histologic grade III, usually the worst prognostically, and they display a high degree of aneuploidy and typically lack hormone receptors. Yet patients with MC often do better than predicted for size and grade [4]. Tumor is infiltrated and surrounded with lymphocytes and plasma cells; in its most exuberant expression, it was classically designated "medullary carcinoma with lymphoid stroma", prompting the oft-stated expression that this tumor may be regulated by a host immune response.

Plasma cells are not themselves cytotoxic and cannot be the direct mediators of tumor suppression or moderation in MC. Rather it is more likely that the antibody products of such plasma cells play this role. Plasma cells are the most mature stage of B cell development with commitment to production of a mature (mainly non-IgM) type of immunoglobulin [5]. B cells directly stimulated by binding of antigen in the presence of T cell help are induced to undergo selection, affinity maturation and class switch. Plasma cells remain in tissue without further cell division. At the site of a vaccination or cutaneous viral infection, specific immune responses include recruitment and maturation of reactive B cells and eventual local generation of plasma cells secreting antibodies directed at antigenic epitopes of the immunizing agent. The persistence of plasma cells at these inflammatory sites, and in MC, is therefore plausibly related to their continued generation from reactive B cells in response to locally concentrated protein neo-antigen.

These newly expressed or modified proteins may be etiologic in the malignant proliferation or they may be markers for the malignancy. The antibodies produced endogenously in these tumors may

interrupt the function of these proteins, or target them as tumor markers, and may be responsible for a more indolent clinical course. Therefore, identification of new tumor-related proteins may reveal new activities in breast carcinoma cells that will enable new therapies tailored to the biology of these proteins.

BODY.

1. Library construction. To date we have successfully constructed two combinatorial phage libraries displaying the antigen binding fragment (Fab) of immunoglobulin (γ_1, κ and γ_1, λ) from two donors with MC according to published methods [6] plus further modifications developed in this laboratory [7]. Both libraries contain more than 10^6 individual members, well in excess of what we will need for effective representation.

Heavy chain				Kappa light chain			Lambda light chain		
Clone	VH gene	D gene	J gene	Clone	Vk gene	J gene	Clone	Vλ gene	J gene
repeated clones				repeated clones			repeated clones		
1	VH251	D3	JH3b	1	Humkv325	JK1	1	Y79	JL2
2	VH251	D3	JH3b	2	Humkv325	JK1	2	Y79	JL2
3	VH251	D3	JH3b	3	Humkv325	JK1	3	Y79	JL2
4	VH251	D3	JH3b	4	Humkv325	JK2	4	Y79	JL2
5	VH251	D3	JH3b	5	Humkv325	JK5	5	Y79	JL2
6	VH251	D3	JH6a				6	Y79	JL2
7	VH251	D21-10	JH3b	6	A2	JK1	7	Y79	JL2
8	VH251	D1	JH4b	7	A2	JK1	8	Y79	JL2
9	VH251	D2	JH4b	8	A2	JK1	9	Y79	JL2
10	VH251	DXP4	JH4b						
11	VH251	D4	JH4b	9	NALM-6	JK1	10	V2.1	JL2
				10	NALM-6	JK1	11	V2.1	JL2
12	VH32	D1rc	JH6b				12	V2.1	JL2
13	VH32	D1rc	JH6b	11	Vg	JK1			
14	VH32	D1rc	JH3b	12	Vg	JK4	13	III.1	JL2
15	VH32	D2rc	JH4b				14	III.1	JL2
16	VH32	D4-brc	JH4b				15	III.1	JL2
17	V5-51	D23-7	JH4b						
18	V5-51	D1rc	JH4b						
19	V5-51	DK4	JH4b						
20	V5-51	D21-9rc	JH3b						
21	V1-02	D3	JH4b						
22	V1-02	D3	JH4b						
23	V1-02	D3	JH4b						
24	V1-18	DK1	JH4b						
25	V1-18	DK1	JH4b						
26	V1-18	D21-7	JH4b						
non-repeated clones				non-repeated clones					
27	V3-11	DK1rc	JH6b	13	JIV	JK1			
28	V71-2	DN1	JH6b	14	Vb	JK4			
29	V4-31	DK1	JH4b	15	Va'	JK4			
30	KIM 13.1	D2	JH3b						

Table 1. Characteristics of H and L chain genes of random clones of a phage display library derived from a patient with medullary ductal carcinoma.

2. Random clone sequencing. In a library of high diversity, there is no repetition of clones in any practical sized sample. In hyperimmunized individuals, antitetanus antibodies were present in a total B cell library in only 1:1000 to 1:5000 clones. Only 2/8 selected *positive* clones that were sequenced showed the same V-gene usage [8]. In a further study with influenza-immunized mice, a dominant H chain and a dominant L chain were present in maximum proportions of 1:200 and

1:1000 in a total B cell library, respectively [9]. Hence, any recurrence of genes in a limited random sample will signal that the library is highly focused. To ascertain the complexity of the MC libraries, the V_H, V_κ, and V_λ sequences of several clones from the two *unselected* MC libraries were determined. Both libraries show a dramatic reiteration of germline sequences (Table 1) [10,11], supporting a focused immune response in the tumor from which the plasma cells originated. Since both libraries show prevalence of different germline genes, these findings are not the result of differences in efficiency of amplification of the IgG heavy and light chain genes during library construction.

3. Selection of phage-Fab clones reactive with malignant breast tissue.

3a. Library panning against MC cells. Using a model system, we have established optimal conditions for cell panning [12]. However, binding studies of both MC phage-Fab libraries to HTB24 cells have yielded inconsistent results. We have been restricted to the use of the only available MC cell line (HTB24) in our panning experiments due to limited availability of fresh primary tumor material. Recently, we have started using MC cells obtained from a mouse MC xenograft (gift from Dr. K. Grabstein, Corixa). Although it is anticipated that this MC tumor has undergone several changes during passaging, we believe that this mouse model is a far better source of MC cells than HTB24, which was established more than a decade ago.

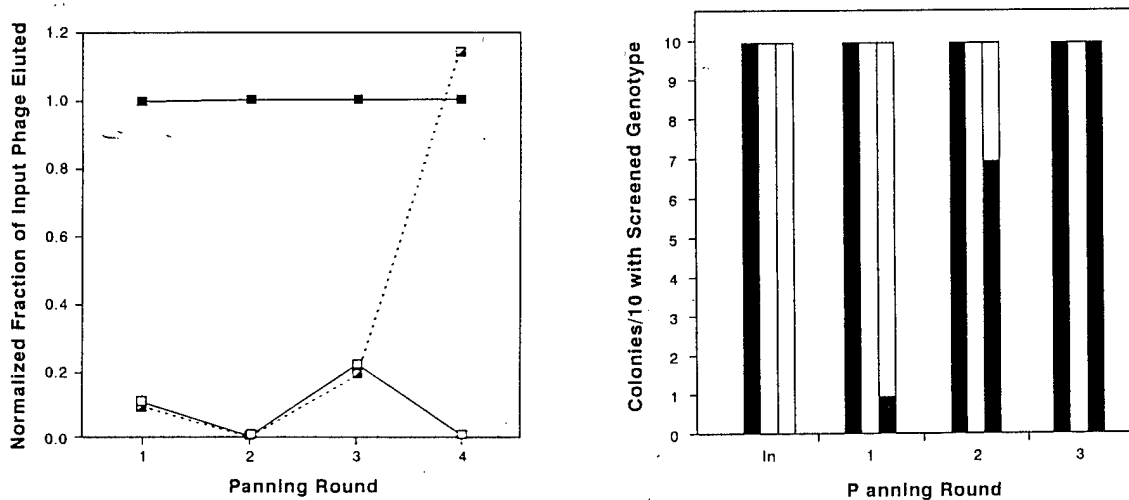


Fig. 1 Enrichment of specific phage Fab by panning with cells displaying mouse-anti-Tac on their surface.
a) Enrichment as indicated by phage elution titers. Elution titers were determined after each round of panning. All elution titers are normalized for the positive controls (anti-Id, ■) in each assay. By the fourth round of panning, elution titers for the 1:1000 mixture (■) of specific phage (anti-Id) and non-specific phage (TT) equal those of the positive control. Negative control (TT, □).
b) Enrichment of specific phage Fab by panning as indicated by HaeIII fingerprinting. First two bars are: specific phage (anti-Id, ■), non-specific phage (TT, □). Third bar represents enrichment in a 1:1000 mixture of specific phage (anti-Id) and non-specific phage (TT). The first set of bars (In) depicts the fingerprints of the initial 1:1000 mixture.

In the model system we have shown that phage titer enrichment is not as accurate as 'HaeIII fingerprint' analysis of eluted clones (Fig. 1). Digestion of heavy and light chain genes from eluted clones with HaeIII restriction enzyme demonstrated considerable enrichment of specific phage-Fab over non-specific phage-Fab, whereas phage titers were still at negative control levels. Therefore we have started including 'HaeIII fingerprinting' as an additional endpoint to check for enrichment after panning.

3b. Single cell V gene cloning. In an alternative approach to cell panning, we are performing V gene cloning from single plasma cells in MC tissue. This is prompted by the extremely focused V gene repertoire proven in the random sequences in two patients examined to date, which makes it likely that relevant reactive clones will be identified in a small number of plasma cells. In a model

system, using tonsil tissue as a source of plasma cells, we have successfully isolated single plasma cells by binding these cells to anti-CD38 coated beads, which can be picked with a glass pipet tip. Following the determination of the optimal RT-PCR conditions for immunoglobulin heavy and light chain genes from plasma cells, these techniques will be applied to the stored MC tissue from which libraries were derived. After demonstrating the focused repertoire of heavy and light chain genes we expect only a limited number of heavy and light chain pairings to be relevant for the in vivo situation.

4. Protein antigen identification. A report by Colnaghi and co-workers [13] showed Her2/neu reactivity of EBV-transformed patient peripheral B cells when patients' tumors (i) overexpressed Her2/neu and (ii) were infiltrated. It is noted, however, that in 4/4 MC tumors tested, there was no Her2/neu overexpression [14; M. Press, pers. comm.], and it therefore seems unlikely that Her2/neu is in fact the principal eliciting antigen that gives MC its characteristic plasma cell infiltrates. Nevertheless, we pursue this for ease of performance and as a further approach to complement the primary methods. We have recently obtained purified recombinant Her2/neu^{ECD} protein [15] for this purpose (gift of Dr. B. Fendly, Genentech). As reported previously several

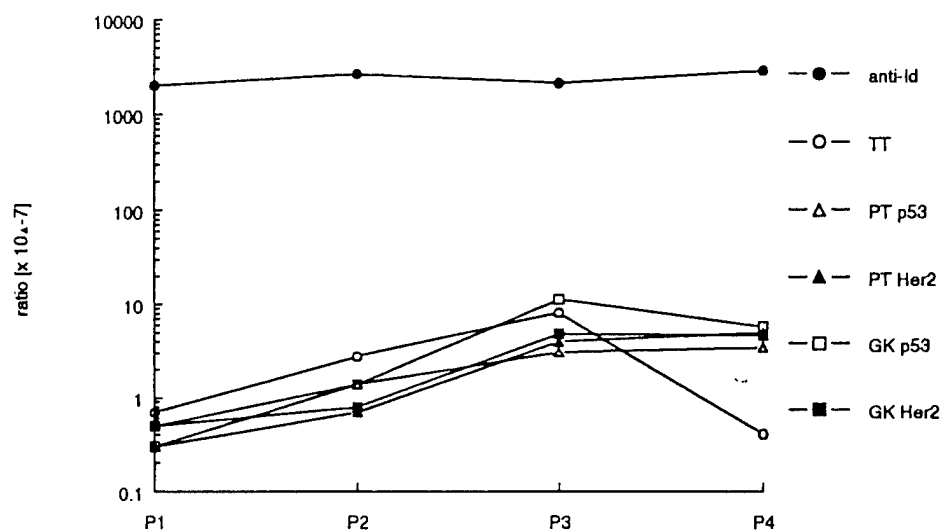


Fig. 2 Panning of two MC phage display libraries against Her2/neu and p53. After coating of an ELISA plate with Her2/neu or p53 the wells were incubated with phage from the two MC libraries. For both positive and negative controls, an ELISA plate was coated with mouse-anti-Tac and incubated with specific phage (anti-Id) or non-specific phage (TT). After incubation bound phage were eluted from the wells, replicated and used in additional rounds of panning. Binding is expressed as the ration of bound phage to input phage.

$\gamma 1, \kappa$ clones and the whole IgG κ and IgG λ libraries were tested in a Her2/neu ELISA, and none was reactive. To rule out the presence of any Her2/neu-reactive clone in either library, panning of both MC libraries against Her2/neu was performed (Fig. 2). The result confirmed our previous conclusions that Her2/neu is not the eliciting antigen in MC.

A portion of breast carcinomas with mutated p53 may have serum antibodies to p53, but against non-mutated regions of the protein [16,17]. No data specifically tie this phenomenon to reactions in plasma cell infiltrated MC or NOS tumors. p53 is normally a nuclear protein, and does not fit our profile for a surface-expressed antigen. However, as stated for Her2/neu, reactivity to p53 is readily tested. We have obtained purified GST-p53 fusion protein (gift of Dr. Dutta, HMS), to establish an ELISA for this test. Similar to Her2/neu, none of the individual clones tested demonstrated affinity for p53. Additionally, panning of both libraries against p53 did not reveal any p53-reactive clones in either library (Fig. 2). p53 is therefore unlikely the eliciting antigen in MC.

5. Development of mouse models and MC cell lines. Until recently our source of MC material was the only available MC cell line HTB24. Although several MC biopsies have been procured from local hospitals and the Eastern division of the Cooperative Human Tissue Network, the amount of tumor that was obtained did not allow us to perform all the experiments we had planned. We have obtained a mouse MC xenograft (gift from Dr. K. Grabstein, Corixa) which was established by using a recently published protocol that greatly enhances the possibility of propagating human breast cancer tissue in mice. This mouse model is maintained by implanting Tudor pieces into the large gonadal fat pad of female NOD-SCID mice (gift from Dr. L. Schultz, Jackson Lab.)[18]. In an effort to develop a MC cell line, pieces of Tudor which were excised from the mouse were placed in cell culture (in collaboration with Dr. V. Band, Tufts Med. School). Cells, which have been in culture for over 2 months now, are currently being analysed for cytokeratin 19 expression and R123 retention, indicative of malignant epithelial cells [19,20].

CONCLUSIONS.

Sequence analysis of unselected clones from both MC libraries clearly demonstrated a highly restricted antibody diversity as expected from a limited, specific response within the tissue. Both libraries show no affinity for the proteins Her2/neu or p53, which are both involved in breast cancer, indicating that these proteins are not responsible for plasma cell infiltration in MC.

Cell panning of the MC libraries with HTB24 cells, the only available MC cell line, gave inconsistent results. After obtaining a mouse MC xenograft we have started using MC tissue in panning experiments. We believe that this source of MC cells is far more reliable than HTB24. Alternatively, we are trying to identify the original heavy and light chain pairing in the MC tumors by single plasma cell PCR.

We have made considerable progress in procuring MC tissue with the help of the Eastern division of the Cooperative Human Tissue Network. The mouse MC xenograft, which as recently be obtained, provides enough material to construct a cDNA expression library of this MC tumor. Purified Fab from selected clones will be used to screen this library to isolate the antigen of interest.

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In preparation.

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